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(57) Abstract

Methods of detecting and/or diagnosing intimin–expressing microorganisms such as enteropathogenic *Escherichia coli* (EPEC), enterohemorrhagic *E. coli*, *C. rodentium* and/or RDEC–1, as well as kits for use in such methods are provided. In particular, methods based antisera raised against conserved regions are described, as well as polypeptide regions useful in the production of antisera. Vaccines based on such peptides are also described herein. In addition, methods of typing/classification of such bacteria are also described herein. Finally, methods for the isolation of intimin–expressing microorganisms are also provided.

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METHODS

The present invention relates to methods of detecting and/or diagnosing intimin-expressing microorganisms such as enteropathogenic *Escherichia coli* (EPEC), enterohemorrhagic *E. coli* , *C. rodentium* and/or RDEC-1, as well as kits for use in such methods. Primers for use in such methods, as well as polypeptide regions useful in the production of antisera are also provided. Vaccines based on such peptides are also described herein. In addition, methods of typing/classification of such bacteria are also described herein. Finally, methods for the isolation of intimin-expressing microorganisms are also provided.

Enteropathogenic *Escherichia coli* (EPEC) are a major cause of acute and persistent infantile diarrhoea in the developing world (33). Traditionally, EPEC strains are considered to belong to twelve different O serogroups: O26, O55, O86, O111, O114, O119, 0125, O126, 0127, O128, O142 and O158 (48). Population genetic surveys, using multi-locus enzyme electrophoresis, have shown that the classical EPEC strains are diverged into two major groups of related clones, designated EPEC clone 1 and EPEC clone 2 (39, 47). Within each group, a variety of O antigens are present while the somatic flagellar (H) antigens are conserved. Strains belonging to EPEC clone 1 typically express H6 and H34, whereas EPEC clone 2 strains express H2 (39, 46).

Small bowel biopsies of children infected with EPEC reveal discrete colonies of bacteria attached to the mucosa (45). Binding of EPEC to the brush border triggers a cascade of trans-membrane and intracellular signals leading to cytoskeletal reorganisation and formation of a specific lesion, termed the "attachment and

effacement" (A/E) lesion (36). This lesion is characterised by destruction of microvilli and intimate adherence of bacteria to cup-like pedestals formed by the bare enterocyte cell membrane (28). High concentrations of polymerised actin are present in the enterocyte beneath the site of bacterial attachment (29). Infection of cultured epithelial cells by EPEC not only induces A/E lesions morphologically similar to those seen in biopsies, but also produces a characteristic pattern of adherence, termed localised adherence (LA) (41). A/E lesions are also induced by other enterobacteria, including enterohemorrhagic *E. coli* (EHEC), the causative agent of bloody and non-bloody diarrhoea as well as of haemolytic uraemic syndrome in humans (40, 43), *Hafnia alvei* which has been isolated from children with diarrhoea (3), *Citrobacter rodentium*, the causative agent of transmissible colonic hyperplasia in laboratory mice (4, 42) and rabbit-specific EPEC strains including rabbit diarrhoeagenic *E. coli* (RDEC-1), which cause diarrhoea in rabbits (8)

Experiments with cultured epithelial cells have implicated several genes in LA and A/E lesion formation by EPEC. These genes map predominantly to two sites. The first is a 35 Kbp pathogenicity island termed the locus of enterocyte effacement or "the LEE region" (26, 35). This locus, found in all A/E lesion forming bacteria, (35) encodes a type III secretion system (22), a series of secreted effector proteins (EPEC secreted proteins or Esps) (12, 27, 32) and intimin, product of the *eaeA* gene (23, 24) that mediates intimate bacterial adhesion to epithelial cells and is required for full virulence in volunteers (13, 14). The second is the ca. 90 Kb EPEC-adherence-factor (EAF) plasmid, common to all typical EPEC strains (25, 38). The EAF plasmid encodes bundle forming pilus (Bfp) protein, which plays a role in LA and facilitates formation of the A/E lesion (11, 18) and contains a regulatory locus (the *per* locus)

(19) that appears to control and co-ordinate expression of several EPEC virulence factors including intimin (19, 30).

The *eaeA* genes of several EPEC and EHEC strains, RDEC-1 and *C. rodentium* and the 3' end of *eaeA* of *H. alvei* have been cloned and sequenced (1, 5, 15, 23, 42, 49). Comparison of the amino acid sequences of the different intimins has revealed that the N-terminal regions are highly conserved, while the C-termini show much less similarity. Nevertheless, two Cys residues at the C-termini are conserved among all the intimin family members. Recently, we expressed the 280-amino-acid C-terminal domain of intimin (Int280), and derivatives of this domain containing N- and C-terminal deletions as maltose-binding-protein (MBP) fusions and tested their cell-binding properties (15, 16). Cell-binding activity was observed only with the MBP-Int280 and MBP-Int150 fusions, localising a cell-binding function of intimin to the C-terminal 150 amino acids (16). Cell-binding activity was abolished when Cys937 was substituted with Ser (16).

We have now produced and characterised polyclonal antisera raised against Int280, expressed as a His-tagged polypeptide, from representatives of EPEC clone 1 and EPEC clone 2 strains O127:H6 (ant-Int280-H6) and O114:H2 (ant-Int280-H2), respectively. Immuno-detection of intimin in whole cell extract using the polyclonal antisera required prior optimisation of the bacterial growth conditions. We found that intimin expression is induced during logarithmic growth at 37°C and that antigenic variation exists within the cell binding domain of intimin expressed by different clinical EPEC and EHEC isolates. Using PCR primers, designed on the basis of the *eaeA* sequences of EPEC strains O127:H6, O114:H2 and O86:H34 and EHEC O157:H7 we have classified the intimin family into five distinct groups.

Thus, in a first aspect the present invention provides a method for detecting intimin expressing microorganisms which comprises the step of bringing into contact a sample which may (or may not) contain such microorganisms with antisera raised against one or more intimins. The antisera can be either polyclonal antisera or monoclonal antisera. In preferred embodiments the one or more intimins is/are derived from EPEC serotype O127:H6 and EPEC serotype O114:H2.

In particular, we have produced antisera against a conserved region, Gly387 to Lys 666, 280 amino acids upstream of the cell binding domain, of *eae* from EPEC strain E2348/69. Antisera against the conserved region is reactive against any of the *eae* strains. Thus, in a second aspect the present invention provides a method for detecting intimin expressing microorganisms which comprises the step of bringing into contact a sample which may (or may not) contain such microorganisms with antisera raised against the Gly387 to Lys666 region of *eae* from enteropathogenic or enterohemorrhagic microorganisms or an antigenic fragment thereof. Thus, for instance, the antisera can be raised against the following polypeptides:

GIDYRHGTGN ENDLLYSMQF RYQFDKPWSQ QIEPQYVNEL RTLSGSRYDL VQRNNNIILE YKKQDILSLN IPHDINGTER STQKIQLIVK SKYGLDRIVW DDSALRSQGG QIQHSGSQSA QDYQAILPAY VQGGSNVYKV TARAYDRNGNSSNNVLLTIT VLSNGQVVDQ VGVTDFTADK TSAKADGTEA ITYTATVKKN GVAQANVPVS FNIVSGTAVL SANSANTNGS GKATVTLKSD KPGQVVVSAK TAEMTSALNA NAVIFVDQTK ASITEIKADK ;

GIDYRHGTGN ENDLLYSMQF RYQFDKPWSQ QIEPQYVNEL RTLSGSRYDL VQRNNNIILE YKKQDILSLN IPHDINGTEH STQKIQLIVK SKYGLDRIVW DDSALRSQGG QIQHGGSQSA QDYQAILPAY VQGGSNIYKV TARAYDRNGN SSNNVQLTIT VLPNGQVVDQ VGVTDFTADK TSAKADGIEA ITYTATVKKN GVAQANVPVT FSIVSGTATL GANSARTDGN GKATVTLKSA TPGQVVVSAK TAEMTSPLNA SAVIFVDQTK ASITEIKADK

GIDYRHGTGN ENDLLYSMQF RYQFDKSWSQ QIEPQYVNEL RTLSGSRYDL

VQRNNNIILE YKKQDILSLN IPHDINGTEH STQKIQLIVK SKYGLDRIVW DDSALRSQGG QIQHSGSQSA QDYQAILPAY VQGGSNIYKV TARAYDRNGN SSNNVQLTIT VLSNGQVVDQ VGVTDFTADK TSAKADNADT ITYTATVKKN GVAQANVPVS FNIVSGTATL GANSAKTDAN GKATVTLKSS TPGQVVVSAK TAEMTSALNA SAVIFFDQTK ASITEIKADK

Which are derived from intimin α, β and γ respectively. The skilled person will appreciate that equivalent regions (ie conserved regions) from other *eae* strains can be used. Preferably, the level of similarity with the above noted sequences will be of the order of at least 75% as determined by a suitable programme such as ClustalW 1.7. Of course, what is essential is that the cross-reactivity is retained.

In a third aspect the present invention provides an isolated or recombinant polypeptide comprising the Gly387 to Lys646 region of *eae* from from enteropathogenic or enterohemorrhagic microorganisms or an antigenic fragment thereof.

DNA sequences coding for such polypeptides form a fourth aspect of the present invention. Such DNA sequences can be provided in the form of vectors which can be used to transform suitable host cells for the production of antigenic polypeptide. Such vectors and host cells form fifth and sixth aspects of the invention respectively.

As described, we are able to classify intimin expressing microorganisms on the basis of their reactivity with antisera raised against the intimin subtypes which we have now found. Thus, in a seventh aspect the present invention provides a method for the classification/typing of intimin containing microorganisms which comprises the step of bringing into contact a sample which may (or may not) contain such microorganisms with antisera raised against one or more intimins

One application of such techniques is in the food industry, where the need for screening for harmful *E.coli* bacteria is becoming of increasing importance. The data described herein therefore provide a method for confidently screening food samples and establishing into which group any intimin-expressing microorganisms found fall. Thus, in an eighth aspect the present invention provides a method of testing/screening a sample of food which comprises bringing into contact the food sample with antisera raised against one or more intimins.

In general, for these aspects of the invention, the methods will also include some form of detection step. The skilled person will appreciate that various means of detection can be used, including ELISA, immunoblot methods etc.

In view of the classification described herein it is also possible to detect/type/classify intimin containing microorganisms by means of PCR using suitable primers, which are tailored to allow amplification of the specific intimins. Thus, in a ninth aspect, the present invention provides a method for detecting intimin expressing microorganisms which comprises the step of amplifying DNA by one or more cycles of PCR from microorganisms contained in a sample using one or more primers which allow amplification of DNA coding for one or more intimins.

Examples of suitable primers for use in such methods include:

5' CCTTAGGTAAGTTAAGT;

5' TAAGGATTTTGGGACCC;

5' ACAAACTTTGGGATGTTC; or

5' TACGGATTTTGGGCAT.

Primers for use in the PCR methods of the invention form another aspect of the invention.

As discussed herein, of particular interest is the conserved region of the intimin proteins. Thus, in an additional aspect, primers useful in amplyfying this conserved region are also provided;specifically:

5' CCG GAA TTC GGG ATC GAT TAC CGT CAT; and

5' CCC AAG CTT TTA TTT ATC AGC CTT AAT CTC.

These primers form tet another aspect of the present invention.

In preferred embodiments of the above-noted aspects of the invention the methods are used for detection/classification/typing of EPEC and EHEC strains.

As described herein, antisera find use in certain methods of the invention. For example, broad spectrum intimin antisera prepared by amplifying a DNA fragment encoding for the conserved region from intimin eg the Gly387 to Lys646 region as exemplified by EPEC strain E2348/69 DNA as a template. The PCR product can then be cloned into a suitable vector and the polypeptide can be over-expressed, purified and used for antiserum production as described herein. Broad reactivity can be confirmed using conventional methods as described herein.Thus, in further aspects the present invention provides antisera raised against EPEC serotype O127:H6 and EPEC serotype O114:H2. In one embodiment the antisera is polyclonal antisera.

As discussed herein, antisera as described herein can be used to detect various serotypes of EPEC and EHEC as well as *Citrobacter rodentium* and rabbit

diarrhoeagenic *E.coli* (RDEC-1). Thus, in another aspect, the present invention provides a method for the detection of EPEC, EHEC, *C.rodentium* and/or RDEC-1 which comprises the step of bringing a sample into contact with antisera, eg polyclonal antisera as defined herein.

In an additional aspect the present invention provides a method for the diagnosis of EPEC, EHEC, *C.rodentium* and/or RDEC-1 infection in a subject, ge in a human or in animal, which comprises the step of bringing into contact a biological sample obtained from the subject with antisera, eg polyclonal antisera as defined herein.

Suitably, the methods of the present invention can be carried out using kits which comprise one or more suitable reagents such as the antisera, eg polyclonal antisera described herein. Such kits form a fifth aspect of the invention.

The PCR methods of the invention can suitably be carried out using kits comprising one or more of primers as described herein as well as optionally one or more reagents for use in carrying out PCR. Such kits form a final aspect of the present invention.

The antisera as described herein can also be used to isolate intimin-expressing microorganisms. Thus, in yet another aspect the present invention provides a method for isolating intimin-expressing microorganisms which comprises bringing into contact a sample which may (or may not) contain intimin-expressing microorganisms with antibodies raised against one or more intimins. The skilled person will appreciate that there are various ways in which such an isolation can be achieved. For instance, the antibodies can be used in the preparation of an immunoaffinity

column, with samples being run through such that intimin-expressing microorganisms will bind thereto. In further aspescts there are provided:

a) the use of antibodies as described herein in the isolation of intimin-expressing microorganisms;

b) an agent for use in isolating intimin-expressing microorganisms comprising antibodies as described herein. For instance the antibody(ies) can be linked to an inert substrate, eg for use in immunocolumn;

c) the use of antibodies as described herein in the preparation of an agent for use in the isolation of intimin-expressing microorganisms.

Finally, the work described herein provides the basis for a vaccine approach to prevention/therapy based on the conserved region of the intimin protein. Thus, in yet further aspects the present invention provides:

i) a vaccine composition comprising one or more polypeptides as described herein;

ii) the use of a polypeptide as described herein in the manufacture of a medicament for the prevention and/or treatment of enteropathogenic and/or enterohemorrhagic microorganisms;

iii) a method for the prevention and/or treatment of infection by enteropathogenic and/or enterohemorrhagic microorganisms which comprises the step of administering an effective amount of a polypeptide as described herein.

Preferred features of each aspect of the invention are as for each other aspect *mutatis mutandis*

The invention will now be described with reference to the following examples, which should not be construed as in any way limiting the invention.

The examples refer to the figures in which:

Fig. 1: shows immunoblotting analysis of polyclonal antisera against various EPEC strains. 0.05 OD of each sample was loaded onto a 7.5% SDS-PAGE and the bacterial cell extracts were reacted with anti-Int280-H6 (A) or anti-Int280-H2 (B). Molecular weight markers (in kilodaltons) are shown in lane 1. Strains E2348/69 O127:H6 (lane 2), O142:H34 (lane 7), O55:H6 (lane 9) and O142:H6 (lane 13) reacted strongly with the anti Int280-H6 while strains O111:H$^-$ (lane 4), O114:H2 (lane 5), O119:H6 (lane 6),O111:H2 (lane 8), O119:H2 (lane 11) and O128:H2 (lane 15) reacted strongly with the anti In280-H2. A weak or no reactivity was observed with both antisera with strains CVD206 (lane 3), O55:H$^-$ (lane 10), O86:H34 (lane 12) and O127:H40 (lane 14);

Fig. 2: shows immunogold labelling of logarithmic phase DMEM grown cultures of EPEC (a-c) and EHEC (d) and immunofluorescence labelling of HEp-2 cell adherent EPEC (e-g) and EHEC strains (h-l) showing: an O127:H6 EPEC strain expressing intimin α (a, e); an O114:H2 EPEC strain

expressing intimin β (b, f); an O86:H34 EPEC strain that expresses neither intimin α nor β (c, g); an O157:H7 EHEC strain expressing neither intimin α nor β (d, h); an O26:H$^-$ EPEC strain expressing intimin β (i) and an O26:H11 EHEC strain expressing intimin β (j) but not intimin α (k). Although not stained with anti intimin α, the phase contrast micrograph of the same field as k shows cell adherent bacteria (l). Panels a-d are x 30,000; panels e-l are x 5500; and

Fig. 3: shows detection of intimin α and intimin β by PCR. Representative strains are shown. PCR products with Int α primer were obtained from E2348/69 (A, lane 2) and from all of the tested O55:H6 (A, lanes 4-9), but with none of the O111:H2 (B, lanes 2-9) or CVD206 (A, lane 3). All the tested O119:H2 (C, 2-7) and O119:H6 (D, 2-7) strains produced a positive PCR product using the Int β primer. In lane 1 of each panel molecular weight markers (1 Kb ladder, BRL) were loaded. The complete DNA analysis is presented in Table 3.

EXAMPLE 1

Bacterial strains

Bacterial strains used in this study included clinical EPEC strains serotypes O127:H6 (E2348/69) (34) and ICC64 (15), O114:H2 (ICC61) (21), O111:H- (B171) (18) and O86:H34 (ICC95); an *eaeA* O127:H6 mutant (CVD206) (10) and the strains listed in Tables 1 and 2. Bacterial strains were grown in L-broth or L-agar. Media was supplemented with 100μg/ml ampicillin or 30μg/ml kanamycin where appropriate. For immunodetection of intimin in whole cell extracts, stationary L-broth cultures

were diluted 1:100 in Dulbecco's modified Eagle's medium containing 2mM L-glutamine (DMEM) and incubated for 3 h at 28 or 37°C.

Preparation of MBP-Int fusion proteins

MBP-Int280 fusion protein from EPEC ICC64 (Int280-H6) was purified as previously described (15). MBP-Int280 fusions from EPEC ICC61 (Int280-H2) and B171 strains, were constructed and purified as described for the other MBP-Int280 fusion protein (15).

Preparation of His-Int280-H6 and His-Int280-H2

In order to express Int280 from ICC64 and ICC61 separately from MBP, the DNA fragments encoding this domain within pMAL-c2 were gel purified, using Prep-A-Gene DNA purification system (Bio rad), after *Eco*RI/*Hin*dIII endounuclease digestions. The fragments were then sub-cloned into a similarly digested pET-28a vector (Novagen Biotechnology) and the recombinant plasmids were transformed into *E. coli* BL21. The His-Int280 polypeptides were purified as suggested by the manufacturer. Briefly, 1 ml of overnight culture of BL21 containing the recombinant pET28a plasmid was inoculated into 100 ml L-broth supplemented with 0.2% glucose and 30µg/ml kanamycin. The culture was incubated for 2 h at 37°C with shaking and expression of His-Int280 was induced by addition of 24mg isopropyl-β-D-thiogalactoside (IPTG). After an additional 4 h incubation at 30°C the cells were harvested by centrifugation, the supernatant discarded, and the pellet was re-suspended in 8 ml binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9) and frozen overnight. The culture was then sonicated at maximum intensity in 10 sec bursts for a total of 3 min with 1 min intervals. The lysate was centrifuged

at 3,200 x g for 30 min and the supernatant was loaded onto a 2.5 ml bed volume pre-washed nickel column.

After loading of the cell extract the column was washed with the following solutions - 25 ml binding buffer, 7.5 ml wash buffer 1 (30 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9) and 7.5 ml wash buffer 2 (60 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9). The bound protein was eluted with 15 ml elute buffer (500 mM imidazole). The fractions were analysed on a 10% polyacrylamide gel electrophoresis (see below).

EXAMPLE 2

Preparation of polyclonal sera

Female Sandy half lop rabbits were immunised subcutaneously with 50-100μg of His-Int280-H6 (made from ICC64) or His-Int280-H2 (made from ICC61) in complete Freund's adjuvant. The animals were boosted twice with the same antigen in incomplete Freund's adjuvant with three week intervals before exsanguination.

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulphate (SDS) was performed as described by Laemmli (31). Protein samples and bacterial extracts to be separated were diluted in an equal volume of 2 x sample

buffer (2% SDS [w/v], 2% 2-mercaptoethanol [v/v], 20% glycerol, and 0.01% bromophenol blue [w/v] in 0.0065 M Tris pH 6.8) and boiled for 5 min prior to loading onto 7.5-10% gels. Molecular weights were estimated using Rainbow molecular markers (Amersham). Following electrophoresis the separated proteins were visualised by staining the gel with Coomassie stain or transferred to nitrocellulose membrane.

EXAMPLE 3

Western blotting (Immunoblotting)

Proteins separated by SDS-PAGE were transferred electrophoretically onto nitrocellulose membranes (Schleicher and Schuell) and immunoblotted according to Towbin *et al* (44) and Burnette (6), at 80v for 90 min. The membranes were blocked overnight in 3% bovine serum albumin (BSA), washed three times with phosphate buffered saline (PBS) containing 0.05% Tween 20 (PBST) and then reacted with the anti-serum of interest for 2 h. Anti-Int280-H6 and anti-Int280-H2 sera were diluted 1:750 and 1:5000 respectively in PBST containing 0.1% BSA. After three washes with PBS the bound antibodies were reacted with horse radish peroxidase-conjugated swine anti-rabbit (1:1000 dilution [DAKO]) and the membranes were developed with hydrogen peroxide and 3'3'-Diaminobenzidine (Sigma) .

ELISA

Briefly, 96 well EIA/RIA plates (Costar) were coated overnight at 4°C with 50µl/well of 2.5µg/ml of Int280 in PBS. The wells were washed three times in PBST and blocked for 1 h at 37°C with PBST-1% BSA. The plates were washed again and then incubated with 5 fold-serial dilutions of the primary antibody in order

to determine the antiserum titre. Two hour incubations with primary and secondary antibodies, diluted in PBST-0.1%BSA, were carried out at 37°C with PBST washes after each step. 50µl of substrate (10 mg o-phenylene diamine tablet [Sigma]) in 12.125 ml 0.1 M citric acid, 12.875 ml 0.25 M $NaHPO_4$ and 10 ml 30% H_2O_2) was added to each well. The enzymatic reaction was terminated by the addition of 12.5% H_2SO_4. The calorimetric reactions were recorded using a Ceres 900 HDi (Biorad-Tek Instruments Inc.) microtitre plate reader. The optical density values were plotted for each sample and the titres were determined as the reciprocal dilution giving an A_{490} of 0.3 above the background.

All titration's and experiments were performed in duplicates. A positive reference serum was used on each plate and the results adjusted accordingly.

EXAMPLE 4

Immunogold labelling of bacterial cells

For immunogold labelling of bacteria, stationary phase L-broth cultures of representative strains were diluted 1:100 in DMEM and grown at 37°C for 4 h. 10µl samples of washed bacterial suspensions were applied to carbon coated grids for 5 min, excess liquid removed, and grids immediately placed face down on drops of anti Int280-H6 or anti Int280-H2 sera (diluted 1:40 in PBS containing 0.2% BSA — PBS/BSA) for 30 min. After thorough washing in PBS/BSA, grids were placed on drops of 10nm gold labelled goat anti rabbit sera (diluted 1:20 [British BioCell International]) for 30 min. After further washing with PBS/BSA and distilled water grids were air dried and viewed under a Jeol 1200EX electron microscope operated at 80kV.

Immunofluorecsence labelling of bacterial cells Immunofluorescence staining was performed on bacteria adhering to HEp-2 cells following a 3 h incubation of HEp-2 cell monolayers with overnight cultures (30). Formalin fixed and washed infected cell monolayers were incubated with anti Int280-H6 or anti Int280-H2 antisera (diluted 1:40) for 45 min. After three 5 min washes with PBS/BSA monolayers were stained with FITC-conjugated goat anti rabbit IgG (Sigma, diluted 1:20) for 45 min. HEp-2 cell preparations were also labelled for cellular actin by simultaneously staining coverslips with a 5μg/ml solution of TRITC-phalloidin (Sigma) (30). Preparations were washed three times with PBS, mounted in glycerol-PBS and examined by incident light fluorescence using a Leitz Dialux microscope. Fluorescence and phase contrast images of the same field were recorded.

EXAMPLE 5

DNA Sequencing of the Int280 from ICC61 and ICC95

The DNA sequence of Int280 from ICC61 was determined from the recombinant pET28a construct and from three independent Taq polymerase (Appligene) PCR products cloned into pGEM-T vector (Promega). The DNA sequence of Int280 from ICC95 was determined from a vent polymerase (New England Biolabs) PCR product cloned into pGEM-T. Primers used were pET28 T7 promoter (5' TTAATACGACTCACTATAGG), pET28 T7 terminator (5' CTAGTTATTGCTCAGCGGT), pGEM-T V1 (5' TGTAAAACGAAGGCCAGT) and pGEM-T V2 (5' ATGTTGTGTGAATTGTG). Plasmids were purified from 4.5ml overnight culture. After centrifugation the bacterial pellets were resuspended in 200μl of 50 mM Tris-HCl pH 7.5 and 10 mM EDTA solution containing 100μg/ml RNaseA. 200μl lysis solution (0.2 M NaOH, 1% SDS) was added before

the mixtures were neutralised with 200µl 1.32 M potassium acetate pH 4.8. Following 5 min centrifugation, the supernatants were extracted twice with 400µl chloroform and the plasmid DNA was precipitated in an equal volume of isopropanol. After washing with 70% ethanol the DNA pellets were dried under vacuum, dissolved in 32l of deionized water and then re-precipitated by addition of 8µl of 4 M NaCl and 40µl of 13% PEG8000. Following 20 min incubation on ice the mixtures were centrifuged at 4°C for 15 min, the pellets rinsed with 70% ethanol, dried under vacuum and resuspended in 25µl of deionized water. DNA sequencing was performed using 0.5-1µg of template DNA and a vector-derived primer using an Perkin Elmer ABI/Prism 377 automated DNA sequencer according to manufacturers instructions. On the basis of the emerging DNA sequence additional (walking) primers were synthesised in the forward and reverse orientations (for sequencing of both DNA strands). Sequence analysis and contig assembly was carried out using Genejockey II in an Apple Macintosh computer. The nucleotide sequence encoding Int280 from ICC61 and ICC95 have been submitted to the GenBank database under accession numbers 111 and 222, respectively.

EXAMPLE 6

Polymerase chain reaction (PCR)

PCR (37) was used to amplify a segment of the *eaeA* gene. Thirty amplification cycles of 95°C 20 sec; 45°C 1 min and 74°C 1 min (except for the Int γ primer for which the annealing temperature was 55°C) were employed. 25 pmol of each of the primers (Table 2) and 1.5 units Taq DNA polymerase (Appligene, Durham, UK) were used. For each reaction, about one third of a colony was transferred to a 0.5 ml tube containing the PCR reaction mixture and primers and the tubes were

incubated at 95°C for 5 min prior to the PCR cycling. 10µl from each reaction were analysed by agarose gel electrophoresis

RESULTS

Immuno-reactivity of anti-Int280-H6 antiserum

The cell binding domain of intimin from EPEC strain ICC64, expressed as a His-tagged polypeptide, was used to raise polyclonal anti-Int280-H6 serum. To find conditions that will enable efficient and reproducible immunodetection of intimin in whole bacterial cell extracts, we conducted a systematic investigation of the levels of intimin expression in cultured ICC64 bacteria. We found, in agreement with previous reports (19, 30), that intimin expression is induced when EPEC is grown to the mid-log growth phase in DMEM at 37°C. In contrast, intimin was undetectable when the DMEM cultures were incubated at 28°C (data not shown). When the rabbit polyclonal antiserum was reacted with Western blots of whole bacterial cell extracts after overnight bacterial cultures had been diluted in DMEM and grown at 37°C for approximately 3h, until the mid-log growth phase was reached, only some of the selected EPEC strains reacted strongly with the antiserum while other strains (including CVD206, an intimin-deficient derivative of E2348/69 [O127:H6]) showed no or weak activity (Table 1). This lack of reactivity could reflect either interbacterial differences in expression levels or antigenic variation within the intimin cell-binding domain expressed by the different EPEC strains.

Preparation of anti Int280-H2 antisera: reactivity of anti-Int280-H6 and anti-Int280-H2 sera with intimin

To investigate the possible existence of antigenic variation within the intimin family of polypeptides, His-Int280-H2 was constructed from a representative of the EPEC clone 2 (ICC61) and used to raise anti-Int280-H2 antiserum in rabbits. Forty one typical EPEC belonging to eight serogroups together with two O55:H7 and seven EHEC strains from widely separated geographical sources (North and South America, Europe and Asia) were analysed by using the anti-Int280-H6 and anti-Int280-H2 antisera. Only some of the EPEC strains (belonging to serotypes O55:H6, O127:H6, O142:H6 and O142:H34), as well as *H. alvei,* reacted strongly with anti-Int280-H6 while the other strains (belonging to EPEC serotypes O55:H$^-$, O55:H7 O86:H34, O111:H2, O111:H$^-$, O114:H2, O119:H2, O119:H6, O127:H40 and O128:H2 and EHEC serotypes O26:H$^-$, O26:H11 and O157:H7 as well as *C. rodentium* and RDEC-1) showed weak or no reactivity (Fig. 1A; Table 1). In contrast, the anti-Int280-H2 antiserum reacted strongly with the strains belonging to EPEC serotypes O111:H2, O111:H$^-$, O114:H2, O119:H2, O119:H6 and O128:H2, EHEC serotypes O26:H11 and O26:H$^-$, *C. rodentium* and RDEC-1. A weak reaction or no reaction was observed with EPEC serotypes O55:H$^-$, O55:H6, O55:H7, O86:H34, O127:H6, O127:H40, O142:H6 and O142:H34 and to EHEC O157:H7 (Fig. 1B; Table 1).

Fig 1 shows immunoblotting of fourteen representatives of these strains (summary in Table 1). No reactivity was observed when the strains were probed with normal rabbit serum (data not shown). These findings show antigenic variation within the cell binding domain and indicate that by using these sera, intimin can be divided antigenically into at least three serogroups (Table 1). These were designated intimin α, recognised strongly by anti-Int280-H6 serum; intimin β, recognised strongly by

anti-Int280-H2; and non-typable (NT), recognised poorly by either antiserum (Table 1).

By using an ELISA with purified MBP-Int280 fusion proteins from different EPEC strains as coating antigens, the degree of cross reactivity of the antisera was quantified. Anti-intimin α antiserum was 100-fold more reactive with MBP-Int280 O127:H6 (ICC64) compared with MBP-Int280 O114:H2 and O111:H$^-$ (ICC61 and B171 respectively). The anti-intimin β antiserum was 10-fold more reactive with MBP-Int280 (ICC61 and B171) than with MBP-Int280 (ICC64). No reactivity was observed with MBP. Comparison of the ELISA titers of the antisera using His-tagged and MBP fusions showed that the presence of MBP had no effect (data not shown). Reaction of the different MBP-Int280 fusion proteins with the polyclonal antisera on Western blots confirmed observation made on whole cell lysates (data not shown). These results further suggest that there are major antigenic differences between intimin α and intimin β.

Immunogold and immunofluorescence labelling of whole bacterial EPEC and EHEC cells

The existence of antigenic variation in different intimins expressed on the bacterial cell surface was confirmed directly by immunogold and immunofluorescence. Both immunogold (Fig. 2a-c) and immunofluorescence labelling of EPEC using anti-intimin α and anti-intimin β confirmed surface intimin expression in logarithmic phase DMEM grown cultures of strains belonging to EPEC Clones 1 (Fig. 2a) and 2 (Fig. 2b) and revealed a uniform distribution of intimin over the bacterial surface: other EPEC strains did not react with either antiserum (Fig. 2c). Strains belonging to serogroups O55:H6, O127:H6, O142:H6 and O142:H34 stained strongly with anti-

intimin α serum, while strains belonging to serogroups O55:H$^-$, O86:H34, O111:H$^-$, O111:H2, O114:H2, O119:H2, O119:H6 and O127:H40 showed weak or no staining (Fig. 2a and e and Table 1). In contrast, EPEC strains belonging to serotypes O111:H$^-$, O111:H2, O114:H2, O119:H2 and O119:H6 stained strongly with anti-intimin β serum and weak or no staining was seen with strains of serotypes O55:H6, O127:H6, O142:H6 and O142:H34 (Fig. 2b and f and Table 1). Weak or no staining with either anti-intimin α or β serum was observed with strains of serotypes O55:H$^-$, O86:H34 and O127:H40 (Fig. 2c and g and Table 1), although complementary fluorescence actin staining and phase contrast microscopy confirmed that the cells were covered with A/E bacteria (data not shown).

Cross reactivity with intimin from EHEC was also examined. Neither anti-intimin α nor anti-intimin β serum stained DMEM grown (Fig. 2d) or cell adherent O157:H7 EHEC strains (Fig. 2h) whereas anti-intimin β, but not anti-intimin α, stained DMEM grown and cell adherent O26:H11 EHEC (Fig. 2j-l) and related O26:H$^-$ (Fig. 2i; Table 1) strains.

Identification of intimin derivatives by PCR

The amino acid sequence of the C-terminal domain of intimin from EPEC ICC61 (O114:H2) and ICC95 (O86:H34) was deduced from the DNA sequence of the cloned domains. Alignment of Int280 from ICC61 (excluding the primer-derived sequence) with the published intimin sequences revealed 50% identity with that of E2348/69 (23); 79.8% identity with Int280 from *C. rodentium* (42); 46.7% identity with O157:H7 (49); 100% identity with RDEC-1 (1), O26:H11 (Genebank accesion number U62656) and O26:B6 (Genebank accesion number U62657); 99.6% identity with O111:H$^-$ (Genebank accesion number U62655) and 47% identity with *H. alvei*

(15). Comparison of Int280 from ICC95 with those of E2348/69 and O157:H7 revealed 49.6% and 46.7% identity, respectively, 47% and 77.6% identity respectively with *H. alvei* and *C. rodentium* while 75% identity with those of *E.coli* RDEC-1 and seotypes O114:H2, O111:H$^-$,O26:H11 and O26:B6 was revealed.

Alignment of the amino acid sequences of intimins α and β revealed several regions of low similarity. On the basis of one such region we synthesised forward DNA primers corresponding to intimins α (Int α) and β (Int β) (Table 3) and tested their ability to distinguish between the two intimin types by PCR. Initially, the Int-R reverse primer, made according to DNA sequences adjacent to the 3' end of the *eaeA* gene, was used (Table 3). 104 classical EPEC, and 27 EHEC-like (7 O26:H11, 4 O26:H-, 6 O157:H7 and 10 O55:H7) isolates were tested.

The results of the DNA analysis, summarised in Table 2, show that all the strains belonging to the serotypes recognised by anti-intimin α serum produced a specific PCR product with the Int α forward primer while all but one of the strains belonging to the serotyes reactive with anti-intimin β serum produced a specific PCR product with the Int β primer. Representative strains analysed by the Int α and Int β primers are shown in Fig. 3. Serotypes that were poorly recognised by both antisera produced no PCR product with either the Int α or the Int β primer.

On the basis of the DNA sequence encoding the cell binding domains of intimin from EHEC O157:H7 (49) and O86:H34 (this study) primers were designed and designated Int γ and Int δ respectively (Table 3). Since primer Int-R would not allow DNA amplification of some NT strain *eae* genes, a new reverse primer (Int-Ru) was synthesised according to the absolutely conserved and universal amino acid sequence WAAGANKY (Table 3). Testing of representative strains with the Int α and Int β

forward primers with Int-Ru reverse primer generated results consistent with those obtained with the Int-R primer. Testing of strains classified as NT in immuno-detection assays by PCR using Int γ and Int δ forward primers together with the Int-Ru reverse primer revealed that all but one of the tested O55:H$^-$, O55:H7 and O157:H7 strains produced a specific PCR product with the Int-γ primer, while the O86:H34 strains produced a specific PCR product with the Int-δ primer. EPEC and EHEC strains expressing intimin α or β did not produce a PCR product with either the Int-γ or the Int-δ primer. EPEC isolates belonging to the O127:H40 serotype produced no PCR product with any of the four forward primers and hence were designated NT. Thus, using a combination of antisera and PCR it was possible to distinguish between five different intimin types.

DISCUSSION

In the above examples we used the cell-binding domain of intimin from two EPEC strains, representatives of EPEC clones 1 and 2, to raise polyclonal anti-intimin sera. Reacting the anti-intimin sera with whole EPEC cell extracts (41 different stains belonging to eight serogroups), revealed antigenic variation within this domain, which seems to be in accordance with the reported diversity in the linear amino acid sequences. Nevertheless, on the basis of the Western blots, the tested EPEC strains could be divided into three groups. The first group consisted strains which reacted strongly with anti-intimin α serum. Importantly, all of these strains which belong to EPEC clone 1 (serotypes O55:H6, O127:H6, O142:H6 and O142:H34) were also positive in PCR using the Int-α primer. The second group included strains that reacted strongly with anti-intimin β serum. These strains (serotypes O111:H2, O111:H$^-$, O114:H2, O119:H2, O119:H6 and O128:H2), with the exception of

O119:H6 (20) all belong to EPEC clone 2 (39, 46) and produced a positive PCR product when the Int-β primer was used.

Seventeen serotype O119:H6 strains were analysed by PCR and all gave consistent results. The third group of strains (serotypes O55:H$^-$, O86:H34 and O127:H40) were recognised poorly by both antisera and produced a PCR product with neither primer Int-α nor Int-β. However, this group of strains, designated NT by immunological criteria could be further classified genetically, using primers designed on the basis of the *eae* sequences from O157:H7 (Int-γ) and O86:H34 (Int- δ). It is necessary to raise antiserum to Int280 from a representative of the NT group of strains to complete the immunological classification.

By using immunological and genetic bioassays we obtained consistent results with all (but 2) of the strains belonging to a specific serotype. In addition, the classification of intimin according to diversity within the cell binding domain, with the exception of O119:H6 and O86:H34 (which although belonging to EPEC clone 1 comprise a different clonal phylogeny (46)) seems to follow the clonal lineages. Significantly, cross reactivity with the anti-intimin β serum was observed with *C. rodentium* and RDEC-1 which also produced PCR products with the Int-β primer while *H. alvei* cross reacted with anti-intimin α serum.

EHEC strains, capable of forming A/E lesions and lacking the EAF plasmid are also divided into two divergent clonal groups (46, 47). EHEC clone 1 includes the serotype O157:H7 clone while EHEC clone 2 composed of shiga-like toxin-producing serotype O26:H11 and O111:H8 strains. Recently, it was shown that serotype O55:H7, an atypical EPEC clone, is closely related to EHEC clone 1 (46, 47). Reacting the anti-intimin sera with representatives of the two EHEC clones

revealed that while strains related to EHEC clone 1 were recognised by neither antiserum, strong cross reactivity was observed with anti-intimin β serum and strains of EHEC clone 2. Similar results were obtained by PCR: while the serotype O26:H11 strains produced PCR products with the Int-β primer, strains belonging to serotypes O55:H7 and O157:H7 produced specific PCR products with the Int-γ primer. Significantly, like O55:H7, the typical EPEC serotype O55:H⁻ was classified by using PCR together with EHEC O157:h7. By using immunogold and immunoflurorescence labelling we have demonstrated the existence of antigenic variation in the epitopes of different intimins expressed on the bacterial cell surface of EPEC and EHEC.

Previously published data from Agin and Wolf (2) and Jerse and Kaper (24) have been brought together to provide proof of the existence of at least three immunologically distinct groups of intimins, ie., those similar to intimins from RDEC-1, EPEC E2348/69 (O127:H6), and EHEC (O157:H7). This cross reactivity did not appear to be serogroup specific. In contrast, our study provides comprehensive evidence, obtained with immune sera, PCR, and a large number of clinical isolates of EPEC and EHEC, of the existence of at least five intimin subtypes which segregated in a serogroup-serotype fashion. An important feature of the antiserum used by Jerse and Kaper is the fact that it was raised using an alkaline phosphatase-intimin fusion, containing the whole conserved N-terminal region of intimin, as the immunogen. This difference may explain in part the differences between the findings of Agin and Wolf and those described herein. In addition, using immunological and genetic bioassays we showed that both *E.coli* RDEC-1 and *C. rodentium* express intimin β. The reason for the lack of cross reactivity between these two intimins as reported by Agin and Wolf is not clear.

An investigation of pathogen-specific factors that protect children from Brazil against diarrheal disease revealed that breastfeeding is protective against EPEC infection. Analysis of colostrum IgA showed that the antibodies reacted strongly with a 94-kDa protein and could prevent the adherence of EPEC to cells in culture (9). Recently, we assayed murine mucosal IgA responses to intimin in the *C. rodentium* model and found that in all the immunologically naive mice that survived initial infection, mucosal IgA antibodies to intimin were detected 28 days post challenge, while no such responses were seen in any of the mice infected with the *eae* mutant of *C. rodentium* (17). Since intimin is highly immunogenic, it is possible that the diversity within the polypeptide cell-binding domain is driven by natural selection. However, it is important to note that despite the high diversity in this region, two stretches of six and seven amino acids (WLQYGQ and WAAGANKY) are identical in all intimins, but are not found in any other sequences in the databases. It is possible, although not yet proven, that these amino acids form part of the binding site. According to the level of the immunological cross reaction between intimins α and β, these conserved amino acids sequences do not seem to be highly immunogenic. However, only our current investigation, aimed at mapping the immunodominant epitopes within Int280, will confirm this experimentally. The high immunogenicity of intimin in infected hosts provides a rational basis to support the concept of engineering an intimin molecule as a basis for an EPEC vaccine.

EXAMPLE 7

Bacterial strains. Bacterial strains used in this example included *E. coli* BL21, clinical EPEC strains serotypes O127:H6 (E2348/69) (34), O111:H- (B171) (18), O55:H7 (ICC57) (this study), an *eae* O127:H6 mutant (CVD206) (10) and the

strains listed in Table 6. Bacterial strains were grown in L-broth or L-agar. Media was supplemented with 100 mg/ml ampicillin or 30 mg/ml kanamycin where appropriate. For immunodetection of intimin in whole cell extracts, stationary L-broth cultures were diluted 1:100 Dulbecco's modified Eagle's medium containing 2 mM L-glutamine (DMEM) and incubated at 37°C for 3 h.

Preparation of broad spectrum intimin antiserum. In order to produce an intimin antiserum reactive with all the intimin types, the fragment encoding the 280 amino acid upstream of the cell binding domain, residues Gly 387 to Lys 646, of *eae* from EPEC strain E2348/69 was amplified (see below) and subcloned into the EcoR1 and HindIII sites within pET28a (Novagen Biotechnology) and the recombinant plasmids were transformed into *E. coli* BL21. pET28a vector directs expression of cloned genes from an inducible T7 promoter as His-tag fusions. Induced cultures were sonicated, the soluble fraction collected and purified on nickel columns. The purity of the polypeptide preparations was confirmed by an SDS-PAGE analysis (see below). Female Sandy half-lop rabbits were immunised subcutaneously with 50-100 mg of the purified intimin antigen in complete Freund's adjuvant. The animals were boosted twice with the same antigen in incomplete Freund's adjuvant at three week intervals before exsanguination.

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulphate (SDS) was performed as described by Laemmli (31). Protein samples and bacterial extracts to be separated were diluted in an equal volume of 2 x sample buffer (2% SDS [w/v], 2% 2-mercaptoethanol [v/v], 20% glycerol, and 0.01% bromophenol blue [w/v] in 0.0065 M Tris pH 6.8) and boiled for 5 min prior to loading onto 7.5-10% gels. Molecular weights were

estimated using Rainbow molecular markers (Amersham). Following electrophoresis the separated proteins were visualised by staining the gel with Coomassie stain or transferred to nitrocellulose membrane.

Western blotting (Immunobloting). Proteins separated by SDS-PAGE were transferred electrophoretically onto nitrocellulose membranes (Schleicher and Schuell) and immunoblotted according to Towbin *et al* (44) and Burnette (6) at 80v for 90 min. The membranes were blocked overnight in 3% bovine serum albumin (BSA), washed three times with phosphate buffered saline (PBS) containing 0.05% Tween 20 (PBST) and then reacted with the anti-serum of interest for 2 h. The broad spectrum intimin antiserum was diluted 1:1000, in PBST containing 0.1% BSA. After three washes with PBST the bound antibodies were reacted with horseradish peroxidase-conjugated swine anti-rabbit (1:1000 dilution [DAKO]) and the membranes were developed with hydrogen peroxide and 3'3'-diaminobenzidine (Sigma).

Polymerase chain reaction (PCR). PCR was used to amplify a segment of the *eae* gene encoding the conserved intimin domain. Thirty amplification cycles of 95 ¤C 20 sec; 55 ¤C 1 min and 74 ¤C 1 min were employed. 15 pmol of each of the primers (table 5) and 1.5 units Taq DNA polymerase (Appligene, Durham, UK) were used. For each reaction, 0.5µl of the overnight cultures was transferred to a 0.5 ml tube containing the PCR reaction mixture and primers and the tubes were incubated at 95°C for 5 min prior to the PCR cycling. Ten ml from each reaction were analysed by agarose gel electrophoresis.

Immunogold labelling of bacterial cells. For immunogold labelling of bacteria, stationary phase L-broth cultures of representative strains were diluted 1:100 in

DMEM and grown at 37 °C for 4 h. 10 ml samples of washed bacterial suspensions were applied to carbon coated grids for 5 min, excess liquid removed, and grids immediately placed face down on drops of anti Int280-H6 or anti Int280-H2 sera (diluted 1:40 in PBS containing 0.2% BSA — PBS/BSA) for 30 min. After thorough washing in PBS/BSA, grids were placed on drops of 10 nm gold-labelled goat anti-rabbit sera (diluted 1:20 [British BioCell International]) for 30 min. After further washing with PBS/BSA and distilled water grids were air dried and viewed under an Jeol 1200EX electron microscope operated at 80kV.

Immuno-fluorescent labelling of bacterial cells. Immuno-fluorescent staining was performed on bacteria adhering to HEp-2 cells following a 3 h incubation of HEp-2 cell monolayers with overnight cultures (30). Formalin fixed and washed infected cell monolayers were incubated with the universal antiserum (diluted 1:40) for 45 min. After three 5 min washes with PBS/BSA monolayers were stained with FITC-conjugated goat anti rabbit IgG (Sigma, diluted 1:20) for 45 min. HEp-2 cell preparations were also labelled for cellular actin by simultaneously staining coverslips with a 5 mg/ml solution of FITC-phalloidin (Sigma) (30). Preparations were washed three times with PBS, mounted in glycerol-PBS and examined by incident light fluorescence using a Leitzs Dialux microscope. Fluorescence and phase contrast images of the same field were recorded.

Results

Production of a the intimin antiserum

We have previously produced intimin antisera directed against the carboxy terminal, cell binding domain, of intimin α and intimin β. Since these reagents were reactive

only with specific subsets of A/E strains, the further aim was to generate a universal intimin antisera reactive with all the intimin types. For that purpose we have cloned in pET28a, following DNA amplification, the 280 amino acid upstream of the cell binding domain, residues Gly 387 to Lys 646, of *eae* from EPEC strain E2348/69 (encoding intimin a). This Int280 region of intimin is highly conserved (Int280-Con) in all the different intimins sequenced to date. The Int280-Con polypeptide was over expressed in *E. coli* BL21, the protein purified on a nickel column and used to raise rabbit polyclonal antiserum. The specificity of the antiserum was confirmed using wild-type (E2348/69) and its *eae* minus derivative (CVD206) on Western blots and Immunogold EM (Fig. 1 and 2).

Detection of intimin by Western blots and PCR

In order to determine the reactivity of the Int280-Con antiserum to react with the different intimin types 77, A/E *E. coli*, wild-type *C. rodentium* and its *eae* minus derivative DBS255, *H. alvei*, *E. coli* K12 (HB101), HB101 expressing the *Yersinia* invasin (HB101(pIR203)) and *Salmonella typhimurium* strain SR11 (Table 1) were examined using Western blots. The bacterial strains were grown to mid-log growth phase in Dulbecco's modified Eagle's medium (DMEM) at 37°C, established growth conditions for optimal intimin expression. Following 3h incubation whole cell lysates, representing equal number of bacteria, were subjected to Western blots. All the *eae*+ strains, reacted with the Int280-Con antiserum. Similar level of reactivity was observed with intimin from strains harbouring the EPEC adherence factor plasmid (EAF), encoding the positive regulator per. Lower reactivity was detected for strains lacking or cured of the EAF plasmid, including B171-4 (not shown) and JPN15 . No reactivity was seen, in addition to CVD206, with DBS255, HB101,

HB101(pIR203) or *S. typhimurium*. This results demonstrate that the Int280-Con antiserum can react with any the *eae*+ bacterial strains and can be used as a broad spectrum (universal) intimin reagent.

We have also screened the entire bacterial collection with the PCR primers made, on the basis of the conserved *eae* region, to amplify Int280-Con. This has produced positive reactivity with 95% of the A/E *E. coli* while none of the non-*E. coli* strain produced a positive PCR reaction (Table 2).

Detection of surface intimin expression by immunogold EM.

In order to determine if the Int280-Con region is exposed on the bacterial cell surface and accessible for binding of the antiserum we have used immunogold EM and live EPEC bacteria. Reacting the Int280-Con antiserum with strains expressing either intimin a, b, or d revealed inter bacterial variation in the number of gold particles associated with individual bacteria . This result is in contrast to the uniform level of reactivity seen on Western blots and indicated variation in accessibility of the antigen.

Discussion

The aim of this study was to develop a broad spectrum intimin antiserum, reactive with all of the intimin types, expressed by A/E lesion forming microbial pathogens. For that purpose we have used a conserved region of intimin located upstream of the cell binding domain. Reacting the antiserum with Western blots of *eae*+ EPEC and non-E. coli strains revealed that the antiserum can recognise any of the intimin types. Similar level of reactivity was observed with all the strains harbouring the Per regulatory proteins, while lower intimin levels were detected on the EAF negative

strains. In contrast, Using immunogold EM we have observed variations in surface exposure of this regions. While on some isolates many gold particles were seen, other strains exhibited only limited accessibility to the antiserum and only low level of gold particle decoration was noticed. A specific PCR product was produced using the PCR primers, synthesised to generate the recombinant intimin epitope, and all the human eae+ E. coli as template. No product was seen with *C. rodentium*, RDEC-1 or *H. alvei*. Accordingly, these PCR primers could be used to specifically detect *eae*+ E. coli strains.

EXAMPLE 8

Preparation of recombinant Intimin. The fragment encoding the 280 amino acid upstream of the cell binding domain, residues Gly 387 to Lys 646, of *eae* from EPEC strain E2348/69 was amplified and subcloned into EcoRI-HindIII sites of pET28a (Novagen Biotechnology). pET28a vector directs expression of cloned genes from an inducible T7 promoter as His-tag fusions. The recombinant plasmids were transformed into *E. coli* BL21. Induced cultures were sonicated, the soluble fraction collected and purified on nickel columns.

Sera. Sera were from 14 patients with faecal E. coli O157 VTEC expressing VT2 only, and with serum antibodies to the O157 LPS antigens. Twenty sera, obtained from apparently healthy individuals, originated from the blood transfusion service, London, UK, were used as controls.

SDS-PAGE/ immunobloting. SDS-PAGE was performed using the method of Laemmli [1970] with an Atto mini-gel system (Genetic Research Instrumentation Ltd.). Preparations containing 10 μg protein in 5μl SDS-PAGE solubilisation buffer were incubated at 100°C (5 mins) prior to loading onto gels comprising a 4.5% acrylamide stacking gel and a 12.5% acrylamide separation gel. Following electrophoresis (50 mAmp, 30 min), gels were either stained with Coomassie brilliant blue or used for immunobloting.

For immunobloting, protein profiles were transferred onto nitrocellulose sheets (0.5 Amp, 1.5 h) using the method of Towbin et al. (44). Following transfer, immunoblots were cut into strips and each profile reacted with patients' sera (10 μl). Antibody-antigen complexes were detected using a goat anti-human polyvalent antibody conjugated with alkaline phosphatase (Sigma Chemical Co. Ltd. UK.), and an enzyme substrate buffer comprising nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate [Chart et al., 1996].

Results

The VTEC-infected human convalescent sera.

Sera were obtained from 14 patients with faecal *E. coli* O157 VTEC expressing VT2 only, and with serum antibodies to the O157 LPS antigens. Nine patients were infected with *E. coli* O157 belonging to phage type 2 and three strains belonged to phage type 21 (Table 1). Seven patients were female (mean age 9.4 (11.7 years) and seven were male (mean age 7.7 (3.6 years). Twelve patients were known to have had HUS (Table 1). Samples of serum and faeces had been referred to the Laboratory of Enteric Pathogens as part of the routine surveillance of VTEC infection in England and Wales.

Reactivity of the human sera with intimin.

Previous reports have shown differential reactivity of human and animal sera with intimin from different VTEC and EPEC isolates. In order to obtain a recombinant intimin antigen reactive with a broad spectrum VTEC-infected human sera, we have cloned in pET28a, following DNA amplification, the 280 amino acid upstream of the cell binding domain, residues Gly 387 to Lys 646, of *eae* from EPEC strain E2348/69 (encoding intimin a), producing Int280$_{387-646}$. The amino acid sequence of this region from EPEC O127 and VTEC O157 is highly conserved sharing 88% identity and 90% similarity. The Int280$_{387-646}$ polypeptide was over expressed in *E. coli* BL21 and the protein purified on a nickel column. On SDS-PAGE, the Int280$_{387-646}$ domain migrated as a band of 45 kDa. Sera from 10 of 14 patients, and none from the healthy controls contained antibodies to this antigen .

Discussion

In the present study, a recombinant preparation of intimin was reacted with sera from patients infected with VT-producing *E. coli* O157. Although other laboratories have made advances in studying the immune response to VTEC by developing assays to detect antibodies to LPS and secreted LEE-encoded VTEC proteins in sera from patients with HUS, this study is to our knowledge the first to use recombinant purified virulance factor to determine antibody responses. For this reason, a rational decision was made to include in this study sera obtained only from culture positive, O157 antibody positive, patients.

Fourteen sera fulfilling this criteria were found. Ten of the patients contained antibodies reactive with intimin, all of which were infected with VTEC O157 belonging to phage type 2. Of the four patients that had no intimin antibodies, three were infected with VTEC O157 belonging to phage type 21. Importantly, none of the 20 control sera contained intimin antibodies.

The isolation and characterisation of EHEC from patients' stools is usually performed to identify the cause of disease, while the detection of *vtx* genes or free VT in faeces can indicate the possible involvement of VTEC. It is not always possible to obtain evidence of EHEC infection using the methods described above, so serological tests based on purified lipopolysaccharide (LPS) have been developed to provide evidence of infection with *E. coli* O157 and other serogroups . In this study we provide further evidence that intimin is expressed during infection with VTEC

and that the conserved region of the outer membrane protein adhesin, may be useful to develop an alternative sero-diagnosis test to VTEC infection.

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Table 1. Summary of Western blotting analysis, and immunogold and immunofluorescence labelling of intimin.

Western blotting		
Int α	Int β	NT[a]
O127:H6 (3/3)[b]	O119:H6 (4/4)	O127:H40 (2/2)
O55:H6 (5/5)	O119:H2 (3/3)	O55:H$^-$ (4/4)
O142:H34 (3/3)	O111:H2 (7/7)	O86:H34 (2/2)
O142:H6 (2/2)	O111:H$^-$ (1/1)	O157:H7 (3/3)
	O114:H2 (2/2)	O55:H7 (2/2)
	O128:H2 (3/3)	
	O26:H11 (4/4)	
	O26:H- (2/2)	
	C. rodentium	
	E.coli RDEC-1	

Immunogold/Immunofluorescence Labelling

Int α	Int β	NT
O127:H6 (2/2)	O119:H6 (1/1)	O127:H40 (1/1)
O55:H6 (1/1)	O119:H2 (1/1)	O55:H$^-$ (1/1)
O142:H6 (1/1)	O111:H2 (1/1)	O86:H34 (1/1)
O142:H34 (2/2)	O111:H$^-$ (1/1)	O157:H7 (3/3)
	O114:H2 (1/1)	
	O26:H11 (2/2)	
	O26:H$^-$ (1/1)	

[a]NT, NT with both antisera

[b] Values in parentheses are numbers of positive isolates/total number of strains tested

5

Table 2: Summary of PCR analysis of intimin derivatives

Serotype, species or strain				
Int-α	Int-β	Int-γ	Int-δ	NT
O127:H6 (6/6)[a]	O119:H6 (17/17)	O55:H$^-$ (18/19)	O86:H34 (2/2)	O127:H40 (2/2)
O55:H6 (15/15)	O119:H2 (6/6)	O55:H7 (10/10)		
O142:H34 (6/6)	O111:H2 (18/18)	O157:H7 (6/6)		
O142:H6 (6/6)	O111:H$^-$ (2/2)			
	O114:H2 (2/2)			
	O128:H2 (3/3)			
	O26:H$^-$ (3/4)			
	O26:H11 (7/7)			
	C.rodentium			
	E.coli RDEC-1			

[a] Values in parentheses are numbers of positve isolates/total number of strains tested

Table 3. Primer sequences used in PCR to classify intimin subtypes

Primer	Position[a]	Orientation	Sequence
Int-F	1	Forward	5'GCCAGCATTACTGAGATTAAG
Int-α	130	Forward	5' CCTTAGGTAAGTTAAGT
Int-β	126	Forward	5' TAAGGATTTTGGGACCC
Int-γ	126	Forward	5' ACAAACTTTGGGATGTTC
Int-δ	125	Forward	5' TACGGATTTTGGGCAT
Int-R[b]	669	Reverse	5' TTTTACACAARYKGCAWAAGC
Int-Ru[b]	868	Reverse	5' TTTATTTGCAGCCCCCCAT

[a] The first G residue within the GCCAGCATTACTGAGATTAAGGCT sequence, encoding the conserved ASITEIKA motif, was defined as nucleotide

[b] The position indicated is that of intimin α (the positions of intimins γ and δ are shifted 16 and 4bp respectively.

Table 4. Summary of PCR analysis of the intimin derivatives

Int α	Int β	Int γ	Int δ	Int ε
O127:H6 (6/6)	O119:H6 (17/17)	O55:H- (18/19)	O86:H34 (2/2)	O127:H40 (2/2)
O55:H6 (15/15)	O119 H2 (6/6)	O55:H7 (10/10)		
O142:H34 (6/6)	O111:H2 (18/18)	O157:H7 (6/6)		
O142:H6 (6/6)	O111:H- (2/2)			
	O114:H2 (2/2)			
	O128:H2 (3/3)			
	O26:H- (3/4)			
	O26:H11 (7/7)			
	C. rodentium RDEC-1			

Table 5: conserved region primers

Primers	
Int-Con-F	5' CCG GAA TTC GGG ATC GAT TAC CGT CAT
Int-Con-R	5' CCC AAG CTT TTA TTT ATC AGC CTT AAT CTC

Table 6: Results of PCR and western blot analysis using primers specific for the conserved intimin region and the anti-Int280(con)α.

(* Numbers include the ab strains, NT none typable strain (Adu-Bodie et al 1998), -/- the figure on the left is the number of positives and on the right the total number tested.)

Serotype, species, or strain	Western blot positive	PCR positive	Intimin type
0142:H6	4/4	4/4	α
0142:H34	4/4	4/4	α
055:H6	8/8	8/8	α
0127:H6*	6/7	6/7	α
0119:H6	8/8	8/8	β
0119:H2	9/10	9/10	β
0111:H2 *	8/8	8/8	β
0111:H-	1/1	1/1	β
0128:H2 *	4/5	4/5	β
026:H11	1/1	1/1	β
026:H-	1/1	1/1	β
055:H7	2/3	2/3	γ
0157:H7	1/1	1/1	γ
055:H-	3/3	3/3	γ
086:H11	1/1	1/1	δ
0114:H-	1/1	1/1	
086:H34	3/3	3/3	δ
0127:H40	4/4	4/4	NT
C. rodentium	1/1	0/1	

C. rodentium (int-)	0/1	0/1
	1/1	0/1
H. alvei		
RDEC-1	1/1	0/1
S.typhimurium (SR11)	0/1	0/1
B171	1/1	1/1
B171-4	1/1	1/1
HB101	0/1	0/1
HB101 (Inv)	0/1	0/1

CLAIMS:

1. A method for detecting intimin expressing microorganisms which comprises the step of bringing into contact a sample which may (or may not) contain such microorganisms with antisera raised against one or more intimins.

2. A method for detecting intimin expressing microorganisms which comprises the step of bringing into contact a sample which may (or may not) contain such microorganisms with antisera raised against the Gly387 to Lys666 region of *eae* from enteropathogenic or enterohemorrhagic microorganisms or an antigenic fragment thereof.

3. A method as claimed in claim 2 wherein the antisera is raised against one or more of the following polypeptides:

GIDYRHGTGN ENDLLYSMQF RYQFDKPWSQ QIEPQYVNEL RTLSGSRYDL VQRNNNIILE YKKQDILSLN IPHDINGTER STQKIQLIVK SKYGLDRIVW DDSALRSQGG QIQHSGSQSA QDYQAILPAY VQGGSNVYKV TARAYDRNGNSSNNVLLTIT VLSNGQVVDQ VGVTDFTADK TSAKADGTEA ITYTATVKKN GVAQANVPVS FNIVSGTAVL SANSANTNGS GKATVTLKSD KPGQVVVSAK TAEMTSALNA NAVIFVDQTK ASITEIKADK ;

GIDYRHGTGN ENDLLYSMQF RYQFDKPWSQ QIEPQYVNEL RTLSGSRYDL VQRNNNIILE YKKQDILSLN IPHDINGTEH STQKIQLIVK SKYGLDRIVW DDSALRSQGG QIQHGGSQSA QDYQAILPAY VQGGSNIYKV TARAYDRNGN SSNNVQLTIT VLPNGQVVDQ VGVTDFTADK TSAKADGIEA ITYTATVKKN GVAQANVPVT FSIVSGTATL GANSARTDGN GKATVTLKSA TPGQVVVSAK TAEMTSPLNA SAVIFVDQTK ASITEIKADK ; or

GIDYRHGTGN ENDLLYSMQF RYQFDKSWSQ QIEPQYVNEL RTLSGSRYDL VQRNNNIILE YKKQDILSLN IPHDINGTEH STQKIQLIVK SKYGLDRIVW DDSALRSQGG QIQHSGSQSA QDYQAILPAY VQGGSNIYKV TARAYDRNGN SSNNVQLTIT VLSNGQVVDQ VGVTDFTADK TSAKADNADT ITYTATVKKN GVAQANVPVS FNIVSGTATL GANSAKTDAN GKATVTLKSS TPGQVVVSAK

TAEMTSALNA SAVIFFDQTK ASITEIKADK;

or a polypeptide having at least 75% similarity therewith.

4. An isolated or recombinant polypeptide comprising or consisting of the Gly387 to Lys666 region of *eae* from enteropathogenic or enterohemorrhagic microorganisms or an antigenic fragment thereof.

5. A nucleic acid molecule coding for a polypeptide as defined in claim 4.

6. A nucleic acid molecule as claimed in claim 5 which is a DNA molecule.

7. A vector comprising a nucleic acid molecule as defined in claim 5 or claim 6.

8. A host cell transfromed with a nucleic acid molecule as defined in claim 5 or claim 6 or a vector as defined in claim 7.

9. A method for the classification/typing of intimin containing microorganisms which comprises the step of bringing into contact a sample which may (or may not) contain such microorganisms with antisera raised against one or more intimins.

10. A method of testing/screening a sample of food which comprises bringing into contact the food sample with antisera raised against one or more intimins.

11. A method as claimed in any one of claims 1 to 10 wherein the antisera is polyclonal antisera.

12. A method as claimed in any one of claims 1 to 11 wherein the method also comprises the step of detectiing interaction between the antisera and one or more intimins.

13. A method as claimed in claim 12 wherein the detection step is an ELISA or an immunoblot method.

14. A method for detecting intimin expressing microorganisms which comprises the step of amplifying DNA by one or more cycles of PCR from microorganisms contained in a sample using one or more primers which allow amplification of DNA coding for one or more intimins.

15. A method as claimed in claim 14 wherein one or more of the following primers is/are used:

5' CCTTAGGTAAGTTAAGT;

5' TAAGGATTTTGGGACCC;

5' ACAAACTTTGGGATGTTC; or

5' TACGGATTTTGGGCAT.

16. A method as claimed in claim 14 wherein the following primers are used:

5' CCG GAA TTC GGG ATC GAT TAC CGT CAT; and

5' CCC AAG CTT TTA TTT ATC AGC CTT AAT CTC.

17. A primer which is capable of hybridising to intimin-α.

18. A primer as claimed in claim 17 which is:

5' CCTTAGGTAAGTTAAGT.

19. A primer which is capable of hybridising to intimin-β.

20. A primer as claimed in claim 19 which is:

5' TAAGGATTTTGGGACCC.

21. A primer which is capable of hybridising to intimin-γ.

22. A primer as claimed in claim 21 which is:

5' ACAAACTTTGGGATGTTC.

23. A primer which is capable of hybridising to intimin-δ.

24. A primer as claimed in claim 23 which is:

5' TACGGATTTTGGGCAT.

25. A method as claimed in any one of claims 1 to 16 wherein the method is for detection/classification/typing of EPEC and EHEC strains.

26. Antisera raised against EPEC serotype O127:H6.

27. Antisera raised against EPEC serotype O114:H2

28. Antisera raised against a polypeptide as defined in any one of claims 2 to 4.

29. Antisera as claimed in any one of claims 26 or claim 28 which is polyclonal antisera.

30. A method for the detection of EPEC, EHEC, *C.rodentium* and/or RDEC-1 which comprises the step of bringing a sample into contact with antisera raised against one or more intimins.

31. A method for the diagnosis of EPEC, EHEC, *C.rodentium* and/or RDEC-1 infection in a subject which comprises the step of bringing into contact a biological sample obtained from the subject with antisera raised against one or more intimins.

32. A kit comprising one or more reagents for use in a method as defined in any one of claims 1 to 16.

33. A kit as claimed in claim 32 comprising antisera against one or more intimins, eg polyclonal antisera.

34. A kit for use in a method as defined in claim 14 or claim 15 comprising one or more primers and optionally one or more reagents for use in carrying out PCR.

35. A kit as claimed in claim 34 comprising one or more primers as defined in any one of claims 17 to 24.

36. A method for isolating intimin-expressing microorganisms which comprises bringing into contact a sample which may (or may not) contain intimin-expressing microorganisms with antibodies raised against one or more intimins.

37. The use of antibodies raised against one or more intiminsin the isolation of intimin-expressing microorganisms.

38. An agent for use in isolating intimin-expressing microorganisms comprising antibodies raised against one or more intimins.

39. The use of antibodies raised against one or more intimins in the preparation of an agent for use in the isolation of intimin-expressing microorganisms.

40. A vaccine composition comprising one or more polypeptides as defined in any one of claims 2 to 4.

41. The use of a polypeptide as defined in any one of claims 2 to 4 in the manufacture of a medicament for the prevention and/or treatment of enteropathogenic and/or enterohemorrhagic microorganisms.

41. A method for the prevention and/or treatment of infection by enteropathogenic and/or enterohemorrhagic microorganisms which comprises the

step of administering an effective amount of a polypeptide as defined in any one of claims 2 to 4.

42. A method for screening the serum of a subject for infection by enteropathogenic or enterohemorrhagic microorganisms which comprises the step of bringing a serum sample from the subject into contact with one or more intimins and/or with a polypeptide as defined in any one of claims 2 to 4.

43. A method as claimed in claim 42 wherein the subject is a human or animal subject.

44. A vaccine comprising a nucleic acid molecule as defined in claim 5 or claim 6.

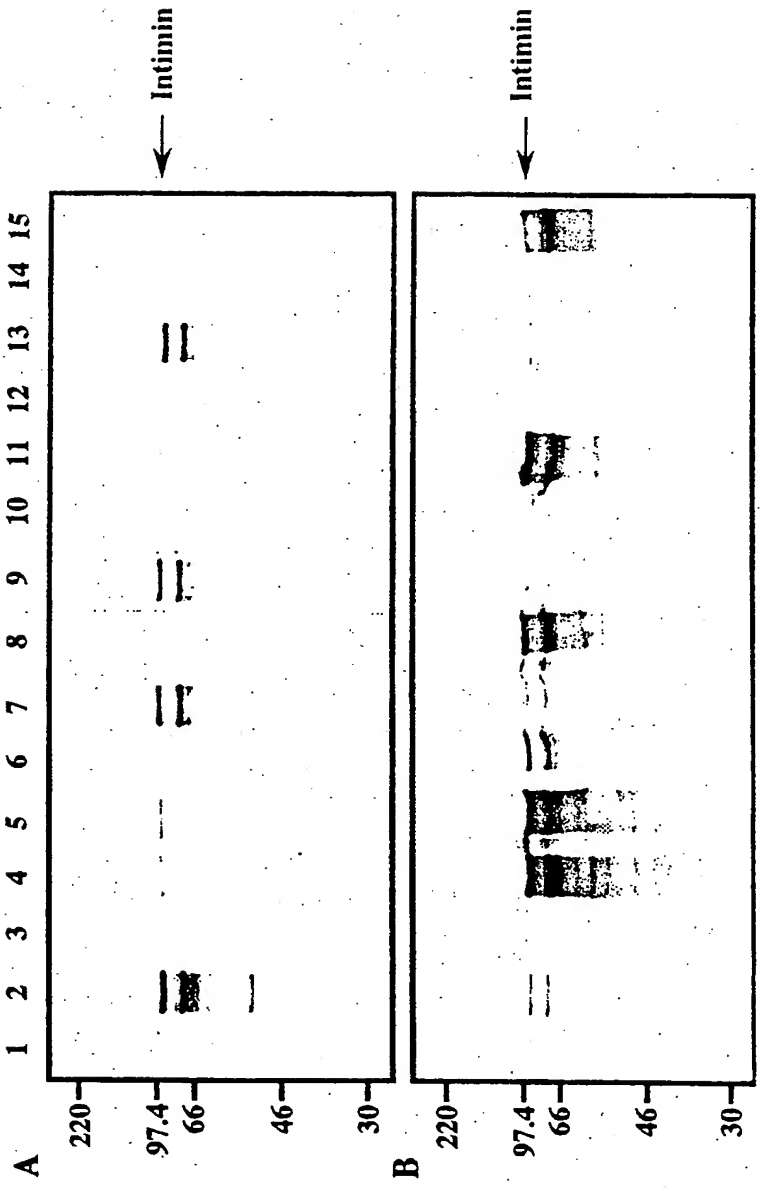
Intimin
Intimin
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
A
B
220
97.4
66
46
30
220
97.4
66
46
30

FIG. 1

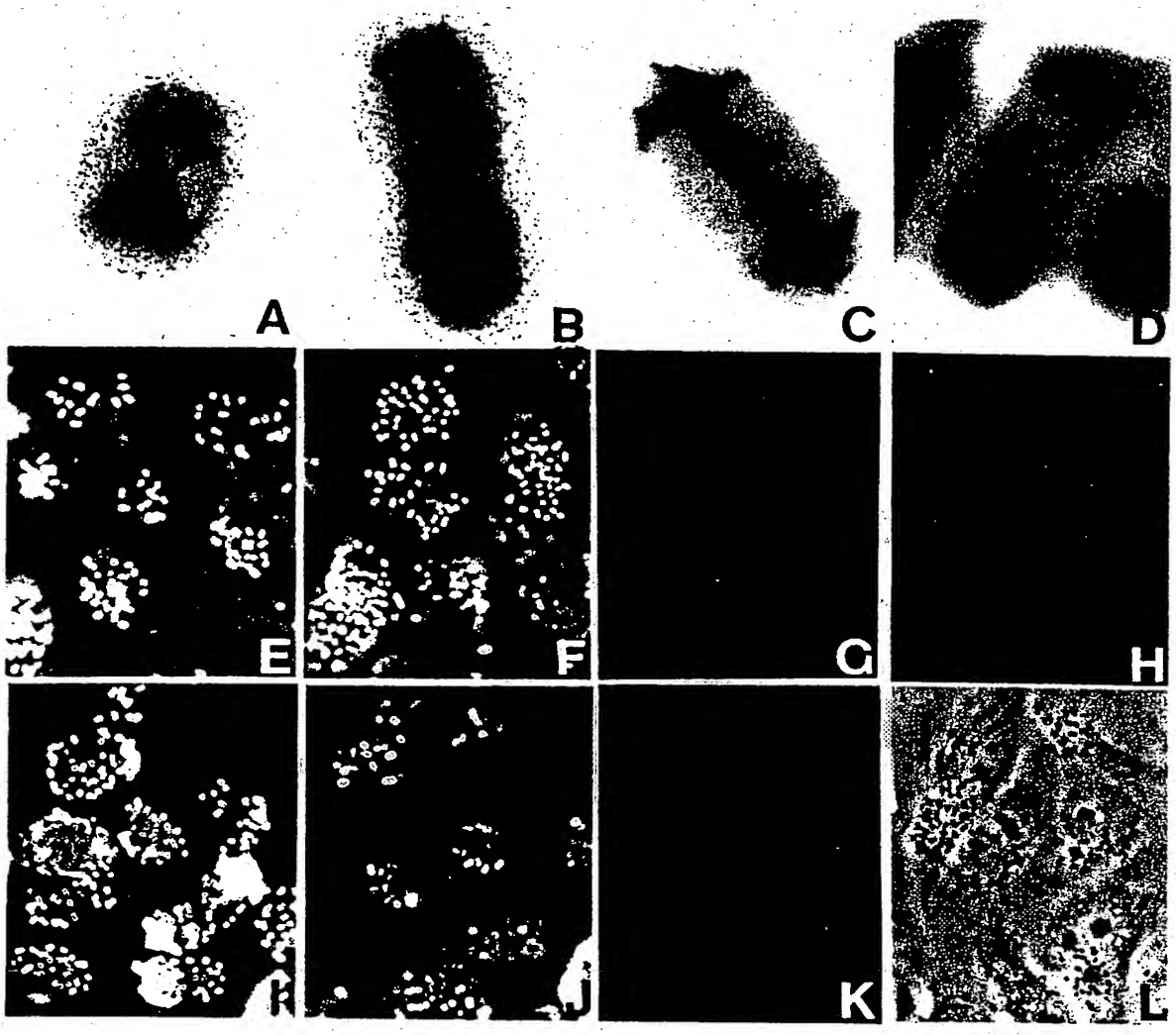

FIG. 2

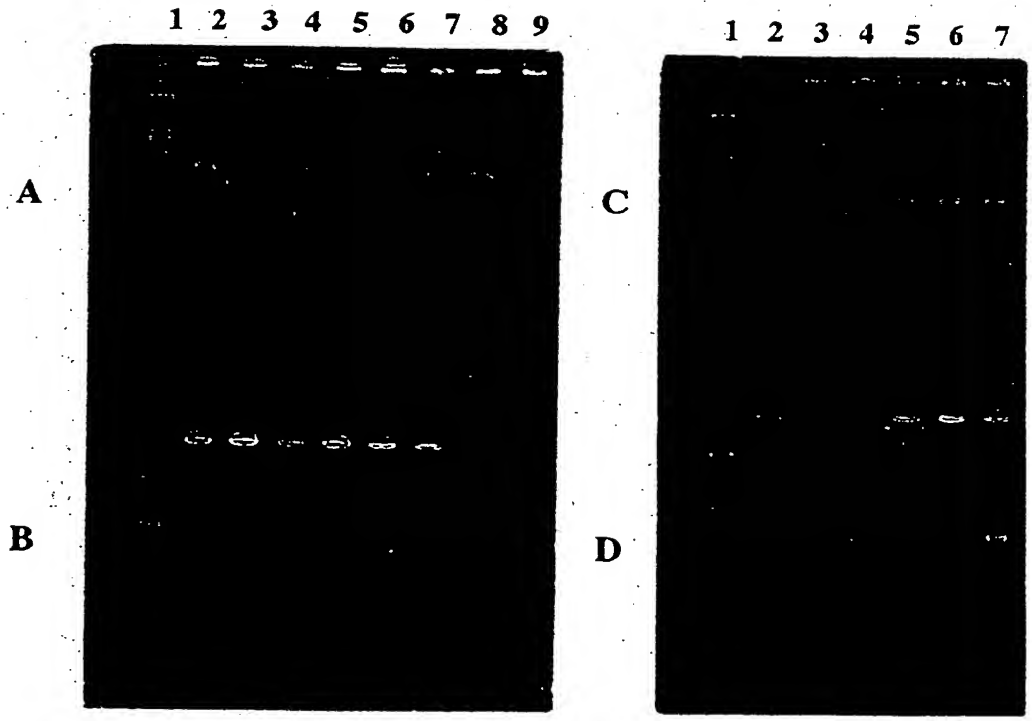

FIG. 3